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# High Endothelial Venules and Other Blood Vessels: Critical Regulators of Lymphoid Organ Development and Function

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The blood vasculature regulates both the development and function of secondary lymphoid organs by providing a portal for entry of hemopoietic cells. During the development of lymphoid organs in the embryo, blood vessels deliver lymphoid tissue inducer cells that initiate and sustain the development of lymphoid tissues. In adults, the blood vessels are structurally distinct from those in other organs due to the requirement for high levels of lymphocyte recruitment under non-inflammatory conditions. In lymph nodes (LNs) and Peyer's patches, high endothelial venules (HEVs) especially adapted for lymphocyte trafficking form a spatially organized network of blood vessels, which controls both the type of lymphocyte and the site of entry into lymphoid tissues. Uniquely, HEVs express vascular addressins that regulate lymphocyte entry into lymphoid organs and are, therefore, critical to the function of lymphoid organs. Recent studies have demonstrated important roles for CD11c<sup>+</sup> dendritic cells in the induction, as well as the maintenance, of vascular addressin expression and, therefore, the function of HEVs. Tertiary lymphoid organs (TLOs) are HEV containing LN-like structures that develop inside organized tissues undergoing chronic immune-mediated inflammation. In autoimmune lesions, the development of TLOs is thought to exacerbate disease. In cancerous tissues, the development of HEVs and TLOs is associated with improved patient outcomes in several cancers. Therefore, it is important to understand what drives the development of HEVs and TLOs and how these structures contribute to pathology. In several human diseases and experimental animal models of chronic inflammation, there are some similarities between the development and function of HEVs within LN and TLOs. This review will summarize current knowledge of how hemopoietic cells with lymphoid tissue-inducing, HEV-inducing, and HEV-maintaining properties are recruited from the bloodstream to induce the development and control the function of lymphoid organs.

**Keywords:** blood vessels, high endothelial venules, peripheral node addressin, mucosal addressin, lymph nodes, tertiary lymphoid organs, ectopic lymphoid structures

## INTRODUCTION

Secondary lymphoid organs (SLOs) are sites in which immune responses are initiated and maintained in order to generate protective immunity against exogenous pathogens and tolerance to self-antigens and commensal organisms (1, 2). These specialized structures include lymph nodes (LNs), Peyer's patches (PPs), tonsils, appendix, bronchus-associated lymphoid tissue (BALT),

nasal-associated lymphoid tissue (NALT), isolated lymphoid follicles, and the spleen. All SLOs develop in the embryo, apart from NALT and BALT, which develop neonatally and in adults, respectively. As well as supplying oxygen and nutrients, the vasculature regulates the development of SLOs by recruiting a distinct population of hemopoietic cells that is essential to initiate lymphoid organogenesis. In adults, the blood vasculature in lymphoid organs is different from that found in other organs due to the requirement for efficient recruitment of lymphocytes under non-inflammatory, homeostatic conditions. Specialized blood vessels called high endothelial venules (HEVs) perform this function in all lymphoid organs except the spleen (3).

Tertiary lymphoid organs (TLOs) are lymph node-like immune cell clusters that develop inside non-lymphoid organs in response to chronic immune-mediated inflammation stimulated by persistent infections, chronic graft rejection, autoimmunity, and cancer (4). Defining features of TLOs are those that define SLOs: HEVs, lymphoid stromal cells, separate T-lymphocyte, and B-lymphocyte-rich compartments, follicular dendritic cell (FDC)-containing germinal centers, and antigen-presenting cells, including dendritic cells (DCs). However, in contrast to SLOs, TLOs are not encapsulated organs and are also known as tertiary lymphoid structures or ectopic lymphoid structures (ELS). TLOs are sites of active immune responses in autoimmune patients and animal models of autoimmunity (5, 6). There is evidence implicating TLOs in generating destructive immunity to self-antigens (7) although the relative contributions of TLOs and SLOs to disease progression are difficult to dissect during ongoing disease. Retrospective studies have correlated the presence of TLOs in resected solid cancers with prolonged patient outcome following resection of the primary cancer in several cancers (8). In some cancers, the density of HEVs alone predicted patient outcome (9, 10), indicating the critical role that HEVs play in orchestrating anti-cancer immunity. Importantly however, the formation of TLOs does not correlate with improved cancer patient outcome for all cancers. In virus-induced hepatic cellular carcinoma, TLOs promote carcinogenesis (11). In *Helicobacter pylori*-infected humans and mice, TLO development precedes carcinogenesis but whether TLOs promote the development of gastric cancer is not clear (12, 13). Therefore, there is much interest in how HEVs and TLOs develop and their exact roles in different chronic inflammatory diseases.

The development of LNs and PPs is well characterized, but how TLOs form is less clear because they develop during ongoing diseases. The similarities in structure between SLOs and TLOs suggest that underlying mechanisms driving their development may be conserved. Much attention has focused on the role of hemopoietic lymphoid tissue inducer (LTi) cells in driving the differentiation of local mesenchyme into lymphoid tissue organizer (LTo) cells during the development of LNs and PPs in mice (14–16). LTo cells are gp38<sup>+</sup> fibroblasts and are precursors of lymphoid stromal cells such as fibroblast reticular cells (FRCs) and FDCs. Lymphotoxin (LT)- $\alpha\beta$  on hemopoietic LTi cells engaging lymphotoxin  $\beta$  receptor (LT $\beta$ R) on LTo cells plays a dominant role in differentiating LTo into chemokine-secreting, adhesion molecule expressing fibroblasts that are able to recruit and retain LTis in the developing lymphoid tissue. An equally important, but

unanswered question, is how embryonic blood vessels deliver LTi cells from their site of generation in fetal liver to predetermined sites where LNs are to develop. A role for inflamed blood vessels in initiating TLO development is implied by the finding that myeloid cells recruited into inflamed tissues drive the differentiation of gp38<sup>+</sup> fibroblasts that share properties with lymphoid stromal cells and are likely precursors of LTo cells (17). However, the mechanisms underlying myeloid cell recruitment by inflamed blood vessels were not determined in this study.

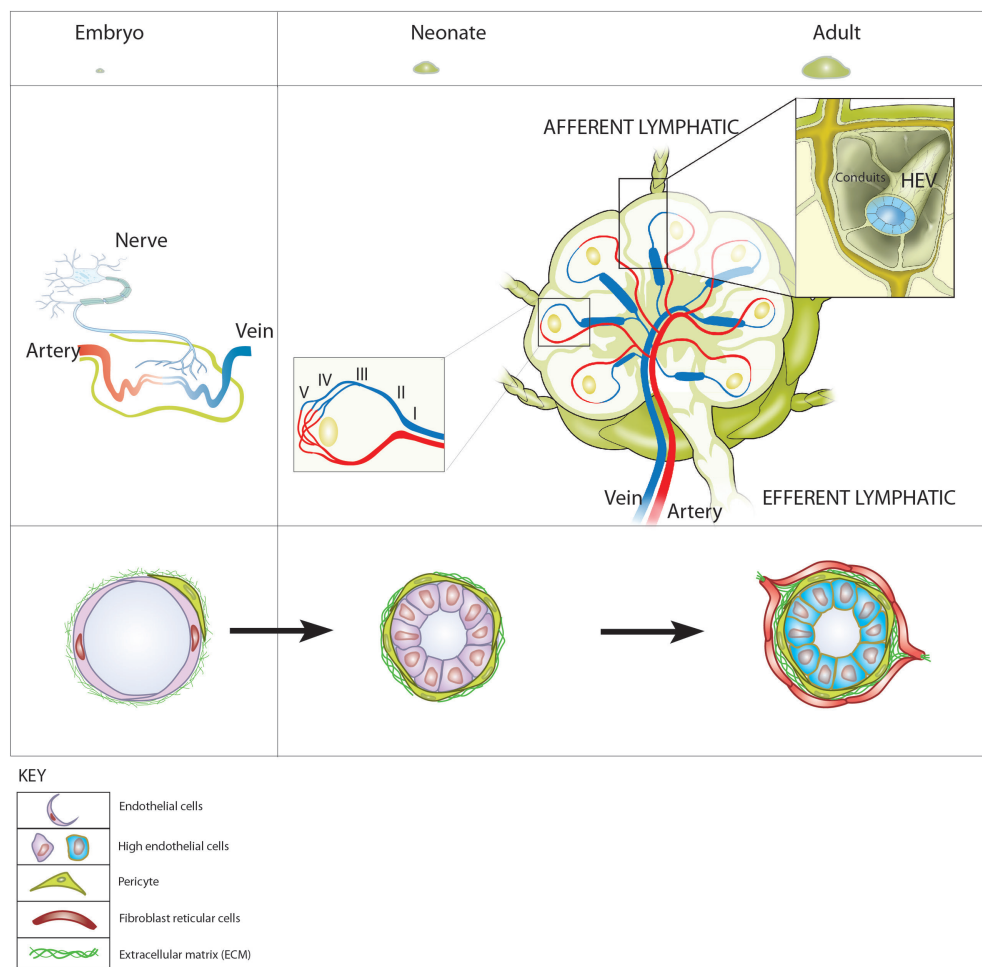
In adults, HEVs continually recruit naive and memory lymphocytes from the bloodstream into lymphoid organs in an antigen-independent manner where they survey DCs for cognate antigen (3). In peripheral LN draining the skin, HEVs express the peripheral addressin, peripheral node addressin (PNAd), whereas in PPs, HEVs express the mucosal addressin, mucosal addressin cell adhesion molecule (MAdCAM)-1. HEVs in some LNs (mesenteric, sacral, cervical) express both PNAd and MAdCAM-1. It has long been known that PNAd expression by HEV is actively maintained since PNAd levels on HEVs are rapidly downregulated following disruption of, or isolation from, the lymphoid microenvironment (18–20). Interestingly, HEVs in peripheral LN of newborn mice express the mucosal addressin but not the peripheral addressin (21, 22). During the first 2–3 weeks of life, HEV maturation is completed by a switch in vascular addressin expression from MAdCAM-1 to PNAd. How the vascular addressin switch is regulated and PNAd expression maintained on HEV in adult SLOs have been unclear. Recent studies have identified critical roles for CD11c<sup>+</sup> DCs in the vascular addressin switch (23) as well as the maintenance of PNAd-expressing HEV in adult mice (24). Therefore, the blood vasculature plays a central role in recruiting distinct populations of hemopoietic cells at precise stages of lymphoid organ development that are essential to initiate lymphoid organogenesis, induce HEV maturation, and maintain fully differentiated HEV. These points will be addressed in greater detail in the following section.

A key step in the recruitment of lymphoid cells is their selection from the total pool of blood-borne leukocytes by binding to the inner blood vessel surface prior to transmigration across the vessel wall and entry into lymphoid organs. This involves a sequence of adhesive interactions between leukocytes and vascular endothelial (VE) cells, which can be divided into distinct stages of rolling and activation-induced arrest. In general, selectins mediate rolling and chemokines immobilized on the endothelial cell (EC) surface activate leukocyte integrins to arrest rolling cells. How hemopoietic cells with LT-inducing, HEV-inducing, and HEV-maintaining properties are recruited into lymphoid organs is central to understanding both the development of lymphoid tissues and the mechanisms regulating adaptive immune responses and disease pathologies.

## BLOOD VESSELS AND THE DEVELOPMENT OF SLOs

The earliest event in LN development is the formation of lymphatic vasculature around embryonic day 10.5 (E10.5) by budding from larger veins and establishing a primordial lymph sac or anlagen (**Figure 1**) (25). CD45<sup>+</sup> CD4<sup>+</sup> CD3<sup>−</sup> IL7R $\alpha$ <sup>+</sup>





**FIGURE 1 | Blood vessels and high endothelial venules (HEVs) in the development of lymph nodes (LNs).** Top: LN development represented as relative sizes of (left) embryonic and (right) neonatal and adult LNs. Middle (left): in the embryo, neuronal stimulation induces retinoic acid-dependent expression of CXCL13 by mesenchymal cells that can be reverse transcytosed and presented on the inner surface of embryonic blood vessels. (Right) In LNs of adults, the HEV network extends from the cortical/paracortical junction adjacent to B cell follicles to large collecting veins in the hilar region gradually increasing in diameter from the smallest, order V venules to the largest order I venules (left hand inset). HEV connect directly to afferent lymphatics via fibroblast reticular cell-coated conduits that form the supporting internal scaffold on which lymphocytes and antigen presenting cells crawl during immunosurveillance (right hand insert). This enables the recruitment of fetal liver-derived CXCR5<sup>+</sup>  $\alpha$ 4 $\beta$ 7 integrin-expressing lymphoid tissue inducer cells to the sites where LN develop. (Right) At birth, structurally distinct MAdCAM-1-expressing HEV lined with cuboidal endothelial cells and supported by a thickened fibronectin containing basal lamina are visible. During the first weeks of life, MAdCAM-1-expressing HEV recruit CD11c<sup>+</sup> neonatal migratory dendritic cells mobilized from the intestinal lamina propria, undergo a switch from MAdCAM-1 to peripheral node addressin expression and become ensheathed by fibroblast reticular cells. The vascular addressin switch is followed by rapid growth and a concomitant increase in cellularity and expansion in LN.

ROR $\gamma$ <sup>+</sup>, Id2<sup>+</sup> lymphoid tissue-inducing (LTi) cells (members of the group 3 category of innate lymphoid cells) are recruited into the anlagen and drive the development of gp38<sup>+</sup> LTo cells from local mesenchyme (26). CXCL13 and IL7, produced by local mesenchymal fibroblasts in response to retinoic acid (RA) generated by neuronal stimulation, recruit CXCR5 and IL7 receptor-expressing LTi cells at sites where LNs are to develop (14). In the embryonic intestine, myeloid cell expression of the tyrosine kinase receptor, RET, is important to localize LTi to sites where PPs develop (27). LT $\alpha$  $\beta$  is upregulated on the surface of incoming LTis and stimulates LT $\beta$ R-dependent upregulation of MAdCAM-1, vascular cell adhesion molecule (VCAM)-1, and

intercellular adhesion molecule (ICAM)-1 and the secretion of homeostatic chemokines CXCL13, CCL19, and CCL21 by lymphoid stromal LTo cells. This enables LTo cells to retain recruited LTi cells in the developing anlagen. The continual recruitment and retention of LTi cells during embryogenesis is required to differentiate sufficient numbers of LTo cells to support the full development and organization of LNs and PPs (22).

During embryogenesis, LTi cells are generated in the fetal liver so how are they recruited to the sites at which LNs and PPs are to develop? A key finding is that all embryonic venous blood vessels express MAdCAM-1 (28). LTis express the integrin  $\alpha$ 4 $\beta$ 7, which binds to MAdCAM-1 and  $\alpha$ 4 $\beta$ 7-MAdCAM-1 binding is

a dominant pathway for leukocyte recruitment from the bloodstream into developing LNs (21). In adults, homeostatic and inflammatory chemokines released by stromal cells are reverse transcytosed and presented on the inner blood vessel surface using Duffy antigen-related receptor-dependent and independent pathways (29–32). By analogy, CXCL13 released by activated fibroblasts presented on the inner surface of embryonic blood vessels together with cell surface MAdCAM-1, recruits CXCR5 expressing LTis from the bloodstream to initiate lymphoid organogenesis. Key questions, therefore, are what controls MAdCAM-1 expression on blood vessels in the embryo and is MAdCAM-1 essential for lymphoid organogenesis? MAdCAM-1 is expressed by all venous blood vessels as early as E9.5 in mice and expression is maintained throughout lymphoid organogenesis (28). In adults, MAdCAM-1 expression by cultured EC is induced by classical Rel A/NF- $\kappa$ B signaling stimulated by factors such as TNF- $\alpha$ , LT- $\alpha$ , IL-1- $\beta$ , and LPS (33–35). Whether constitutive MAdCAM-1 expression on blood vessels in the LN anlagen is driven by NF- $\kappa$ B signaling or is ontologically determined, for example, by the transcription factor NKX2.3 (36), has not been determined. Interestingly, MAdCAM-1 expression by embryonic blood vessels is not essential for lymphoid organogenesis since LNs and PPs develop in MAdCAM-1 ko mice (37). LTis also express  $\alpha 4\beta 1$  integrin, which binds to VCAM-1 and could substitute for MAdCAM-1, however, mice globally deficient in VCAM-1 die in utero (38) and the role of VCAM-1 in lymphoid organogenesis is undetermined. ICAM-1 expression by LTo cells is not essential for lymphoid organogenesis since LNs develop in ICAM-1-deficient mice (39). However, MAdCAM-1, VCAM-1, and ICAM-1 are not redundant in adult mice because they regulate lymphocyte recirculation through SLOs and/or recruitment to inflamed tissues (1, 3).

Lymph nodes and PPs do not develop in LT $\alpha$  ko mice (40). Administration of an LT $\beta$ R agonist to pregnant mice bypasses the requirement for LTi cells and induces LN development (41). The initiation of LN and PP organogenesis is time restricted; mesenteric LN starts around E9–10 and PPs around E16 with peripheral LN (axillary, brachial, inguinal, and popliteal) between E10 and E16. However, exogenous administration of LT $\beta$ R agonists outside of these times will not initiate LN formation. Administration of antagonistic LT $\beta$ R-Ig to pregnant wildtype mice also blocks the development of LN in a similar time-dependent manner (42). What controls the timing of LN development is not clear but one possibility is the ability of embryonic blood vessels to recruit LTi cells. For example, the expression of sufficient MAdCAM-1, immobilized chemokines, or other, as yet unidentified, homing-associated molecules that recruit LTi cells from the bloodstream into the LN anlagen may be time dependent. The local generation of RA drives lymphoid organogenesis and RA is required to mature developing blood vessels in the embryo (43). It is possible that locally generated RA facilitates LTi recruitment by promoting the expression or activity of homing-associated molecules on growing vessels in the LN anlagen, but we know very little about when RA is produced and how this is regulated.

Although LT $\alpha\beta$ –LT $\beta$ R is a dominant NF- $\kappa$ B signaling pathway and CXCL13 a dominant chemokine in the development of peripheral LN and PP, other pathways can substitute during the

development of mucosal and visceral draining LN. For example, mesenteric, cervical (submandibular), sacral, and lumbar LN (44) develop in LT $\beta$ -, LT $\beta$ R-, or CXCL13-deficient mice (15, 22). LT $\beta$ R engagement by membrane LT $\alpha\beta$  stimulates both classical NF- $\kappa$ B signaling *via* I $\kappa$ B and p50/RelA as well as non-classical signaling *via* I $\kappa$  $\alpha$  and p52/RelB, and there is considerable interplay between these two pathways (45). LN and PP do not develop in mice globally deficient in either RelA or Rel B, key components of classical and non-classical NF- $\kappa$ B signaling, respectively, although the impact of classical NF- $\kappa$ B signaling on LN development may be *via* upregulation of non-classical NF- $\kappa$ B signaling substrates such as NF- $\kappa$ B2 and RelB (46, 47). In mice deficient in NF $\kappa$ B2, the substrate for p52 in the non-classical pathway, mesenteric and some peripheral LN develop but lymphoid organs that form later in embryogenesis (inguinal, popliteal, and PPs) are small or do not develop at all (48). It is suggested that p50/RelB can substitute for p52/RelB in these mice but the signal strength is weaker and so, although LTi cells are recruited, the induction of CAMs is not enough to retain sufficient LTi cells to maintain development and organize the full structure of late developing lymphoid organs. Non-classical NF- $\kappa$ B signaling is important for the proper development of HEVs since the small LN that develop in LT $\beta$ - or NF $\kappa$ B2-deficient mice have poorly developed HEVs, as do peripheral LNs that develop in mice expressing a signaling-deficient mutant of the non-classical NF- $\kappa$ B pathway, I $\kappa$  $\alpha$  (48, 49). The widespread expression of LT $\beta$ R on the developing vasculature as well as LTo cells makes it difficult to assess their relative contributions to the development of lymphoid organs or of HEVs in mice globally deficient in either LT $\alpha\beta$ –LT $\beta$ R or following administration of antagonistic LT $\beta$ R-Ig. Recent studies have shown that the development of peripheral LNs in 25–40% of pups is completely blocked in mice selectively deficient in LT $\beta$ R in blood and lymphatic ECs, but the underlying mechanism is not clear (50). Further work will be required to dissect the roles of NF- $\kappa$ B signaling in the blood vs the lymphatic vasculature during lymphoid organogenesis and how this integrates into the scheme of lymphoid organogenesis driven by gp38<sup>+</sup> lymphoid stromal LTo cells.

## THE DEVELOPMENT AND FUNCTION OF HIGH ENDOTHELIAL VENULES IN SLOs

### The Vascular Addressin Switch

The recruitment of naïve T and B cells into all lymphoid organs, apart from spleen, is dependent on the differentiation of a subset of blood vessels into HEVs. Structurally distinct HEVs are not apparent in LN of mice until birth when a branching network of HEV blood vessels starts to organize around B cell follicles during the first days after birth in PPs (28). A key event in neonatal maturation and expansion of LN is a switch in vascular addressin expression by HEV (21). In LNs of newborn mice, all HEVs express the mucosal addressin MAdCAM-1; during the first few weeks of life MAdCAM-1 is downregulated and expression of the PNAd is upregulated. PNAd comprises a mixture of ligands for L-selectin (CD34, podocalyxin, GlyCAM-1, MAdCAM-1, nephrin, and endomucin) that are modified

by 6-sulfo sialyl Lewis<sup>x</sup> on extended core 1 O-linked oligosaccharides and detected by monoclonal antibody MECA79 (51). PNAd expressed on the inner, apical surface of HEVs co-operates with the arrest chemokine CCL21 to select L-selectin/CD62L<sup>+</sup>, CCR7<sup>+</sup> lymphocytes from the bloodstream for entry into LN allowing postnatal colonization of LN by naive T and B lymphocytes as they are released into the circulation from the thymus and fetal liver/bone marrow, respectively. Interestingly, PNAd is also expressed on the basolateral surfaces of ECs lining HEVs but it is not involved in recruiting lymphocytes directly from the bloodstream. Distinct sulfotransferases generate apical and basolateral PNAd and the expression of GlcNAc6ST-2 (HEC-6ST; CHST4), which is required to generate apically expressed PNAd, is restricted to HEV ECs, whereas GlcNAc6ST-1 (CHST2), which generates basolaterally expressed PNAd, is more widely expressed in stromal cells (51). The PNAd-expressing HEV network grows alongside the growth of other stromal components resulting in a rapid increase in the size and cellularity of LN during the first weeks of life (23). Although PNAd is a well-characterized ligand for L-selectin, it only accounts for 50% of L-selectin-dependent recruitment into peripheral LNs. Important non-PNAd ligands on HEV that regulate L-selectin-dependent recruitment into peripheral LN include 6-sulpho sialyl Lewis X modified N-linked glycans such as CD34, as well as a minor role for core 2-branched O-linked glycans (51, 52).

Vascular addressins expressed on the inner, luminal surface of ECs lining HEV bind to homing receptors on lymphocytes; therefore, addressins define the specialized property of HEV in driving lymphocyte recruitment into LNs and PPs. PNAd was originally described in HEVs of peripheral LNs draining the skin (inguinal, axillary, brachial, and popliteal) (53) and MAdCAM-1 in mucosal-associated lymphoid tissues such as PPs (54). However, analysis of other mucosal and viscera-draining SLOs in mice shows that PNAd expression is upregulated and MAdCAM-1 variably downregulated. For example, MAdCAM-1 expression is not completely downregulated in mesenteric and sacral LNs where individual high endothelial cells (HECs) co-express PNAd and MAdCAM-1. However, mucosal addressin is completely downregulated in lymphoid tissues draining other mucosal sites such as cervical (submandibular) LN or NALT (4). Similarly, LNs draining visceral organs, such as lumbar LN, express PNAd and not mucosal addressin (44); therefore PNAd expression is not restricted to subcutaneous LN and is widely expressed by mucosal and visceral LN in mice. PNAd expression was originally reported to be restricted to the basolateral surfaces of HEVs in PPs (54). However, in different mouse strains and other species, PNAd expression in PPs is comparable with that in peripheral LN, which may reflect increased antigenic stimulation (51). Why MAdCAM-1 expression is maintained by HEVs in PPs and some mucosal LN whereas other HEVs completely switch to PNAd expression is not clear, but MAdCAM-1 expression is regulated by the transcription factor NKX2.3 (55).

## The Connection with Afferent Lymph

A unique feature of HEV is the connection with afferent lymph. HEVs are supported by a perivascular sheath composed of FRCs that are connected to the FRC coated conduit system within LN.

The conduit system allows communication between afferent lymph and HEV whereby incoming lymph-borne soluble factors, such as chemokines and cytokines, are delivered directly to the basal lamina of HEV (**Figure 1**) (56). Early studies showed that ligation of afferent lymphatics, which drain fluid and immune cells into popliteal LN, resulted in a gradual flattening of HECs and although some PNAd expression was retained it was restricted to the basolateral surfaces of HECs (18, 19, 57). Other changes in HECs included a transient induction of MAdCAM-1 expression, which peaked 4 days following deafferentization suggesting reversion of mature HEVs to an immature state. Lymphocyte recruitment by HEV decreased and was no longer detectable 8 days after deafferentization. HEVs reverted to fully functional vessels expressing PNAd at the luminal surface following regrowth of afferent lymphatics demonstrating the complete reversibility of HEV differentiation. Early studies indicated a dominant role for LT $\beta$ R signaling in maintaining PNAd expression since chronic administration of LT $\beta$ R blocking reagents to adult mice recapitulates the effects of deafferentization in that HEVs downregulate PNAd and MAdCAM-1, and therefore lose the ability to support lymphocyte homing (58). The flattened appearance of PNAd-positive HEV in deafferentized LNs is seen in peripheral LNs that develop in mice in the absence of endothelial expression of LT $\beta$ R (50) and in inguinal LNs developing in the absence of either NFKB2 or IKK $\alpha$  (48, 49), indicating a key role for LT $\beta$ R stimulated non-canonical NF $\kappa$ B signaling in driving luminal expression of PNAd by HECs. Which cells engage LT $\beta$ R on HECs to induce and maintain PNAd expression has been unclear, however, recent studies identify dominant roles for CD11c<sup>+</sup> DCs in the induction, as well as the maintenance, of PNAd expression by HEV.

## Role of CD11c<sup>+</sup> Cells in the Vascular Addressin Switch

An important finding is that during the first weeks of life CD11c<sup>+</sup>, CD11b<sup>+</sup>, CD103<sup>+</sup> DCs mobilized from the gut lamina propria in response to microbial colonization induce the switch from mucosal to peripheral addressin expression by HEV in peripheral LN (23). RA-dependent signaling is critical for these so-called neonatal migratory DCs to induce the vascular switch; cells that do not express retinaldehyde dehydrogenase (RALDH), which generates RA from retinaldehyde, a derivative of Vitamin A, are unable to induce the vascular addressin switch. This important finding came from studies of adult germ-free mice in which peripheral LN HEVs express MAdCAM-1, but not PNAd, and LN are small without clear compartmentalization into T and B-cell areas, similar to LN in newborn mice. When germ-free mice were co-housed with conventionally housed mice, HEVs underwent the vascular addressin switch with consequent increases in lymphocyte homing and LN cellularity. These effects could be reproduced by administration of RALDH<sup>+</sup> neonatal migratory DCs isolated from conventionally housed adult mice to young germ-free mice. Migration of neonatal migratory DCs to peripheral LN was dependent on MAdCAM-1 expression by HEVs in peripheral LN. Interestingly, the migration of these cells to peripheral LN was highest around 2 weeks after birth and subsided after 6 weeks, presumably because of the loss of MAdCAM-1 expression by peripheral LN HEVs.

In adult mice, CD11c<sup>+</sup> cells have been shown to maintain PNAd-expressing, fully functional HEVs by stimulating LTβR-dependent signaling in HECs (24, 50). Moussion and Girard used transgenic mice expressing the diphtheria toxin (DT) receptor under the CD11c promoter (CD11c-DTR mice) and DT to deplete CD11c<sup>+</sup> cells (24). They noted a gradual loss of PNAd expression, a transient expression of MAdCAM-1, and a reduction in lymphocyte entry *via* HEVs over 8 days, which was prevented by administration of exogenously generated LTαβ-expressing CD11c<sup>+</sup> DCs. Since ligands for LTβR are membrane bound, LTαβ DCs might be expected to make frequent contacts with HECs under homeostatic conditions in order to maintain PNAd expression. Migration of DCs into LN of adult mice has focused on entry from surrounding tissues *via* incoming, afferent lymphatics. DCs entering *via* this route do not come into cellular contact with HECs since they do not penetrate the surrounding pericytic sheath (59, 60). However, DCs entering from surrounding tissues *via* afferent lymphatics do locate around HEVs (61); it is therefore possible that protruding DC membranes or exosomes released by DC (62, 63) could cross the pericytic sheath and contact with HECs. Precursors of classical DCs that are recruited from the bloodstream into LN of adult mice spend up to 5 h in the walls of HEVs are a possible HEV-maintaining cell. In this scenario, the critical role of afferent lymph in maintaining fully differentiated HEVs is not as an entry point for DCs but to supply the chemoattractants necessary to recruit LT-expressing DCs from the bloodstream, enabling DC-HEC contact to sustain LTβR signaling.

Interestingly, CCR7<sup>+</sup> expression by CD11c<sup>+</sup> cells is required to control the homeostatic expansion of PNAd-expressing HEV but in the absence of CCR7 expression, CD11c<sup>+</sup> cells are still able to induce the switch in addressin expression to PNAd (64). The increase in size of the HEV network and associated increase in LN cellularity and volume is due, in part, to the release of angiogenic stimuli such as VEGF-A by FRC and DCs (65, 66). These findings suggest that the differentiation of fully functional, PNAd-expressing HEV and the expansion of the HEV network are controlled by different types of CD11c<sup>+</sup> cells in adult mice. It will be interesting to determine whether RA signaling is required for CD11c<sup>+</sup> DCs to maintain PNAd expression and expand HEV in adult mice as has been shown in neonates (23).

## High Endothelial Venules in Homeostatic Lymphocyte Trafficking

Together, the SLOs are uniquely placed to survey the whole body for incoming pathogens and self-antigens derived from the skin, mucosal surfaces, or directly into the bloodstream, mount an appropriate immune response and clear invading pathogens from the body. To achieve this, rare lymphocytes with appropriate antigen receptor must be selected from the total pool of lymphocytes each with its own unique receptor; this occurs inside SLOs. Pathogens are first degraded and presented as MHC-binding peptides on DCs bound to gp38<sup>+</sup> FRCs. The total pool of lymphocytes in the body is then exposed to pathogen peptide-enriched DCs. Lymphocytes with antigen receptors able to respond to the level of peptide presented are removed from the circulating pool of cells and undergo proliferation and

differentiation to effector, memory, or regulatory lymphocytes before returning to the circulation. Blood vessels are critical components of adaptive immunity since they recruit naïve and central memory lymphocyte irrespective of antigen receptor specificity and deliver them to DCs inside lymphoid organ under homeostatic conditions. Postcapillary venules (PCV) lined with continuous ECs are the preferred sites of lymphocyte extravasation in LNs and other organs such as the skin and GI tract. The increase in diameter as blood vessels transition from capillaries to PCV alters hemodynamics such that leukocytes move to the outer stream or margin of flowing blood adjacent to the inner surface of the vessel (67). Here lymphocytes tether, roll, and arrest on the inside surface without obstructing flow that would happen in smaller capillaries. In other organs where the microvessels are lined with sinusoidal endothelium, such as the spleen, liver, and bone marrow, shear stress at the vessel wall–blood interface may be significantly reduced that the requirement for selectins for capture from flowing blood is not necessary and other homing-associated molecules such as leukocyte integrins perform this role (67). In LNs and PPs, the trafficking of naïve lymphocytes is restricted to specialized postcapillary venules called HEVs. These vessels are structurally adapted to support large-scale lymphocyte trafficking without compromising vascular integrity. The spleen does not have HEV and, in mice, lymphocytes enter the spleen from capillaries in the marginal zone using incompletely defined molecular recognition pathways. The structure of human spleen differs from that in mice and the route of lymphocyte entry has not been identified (68).

Arteries feeding the LN enter at the hilar region and arborize into nutrient- and oxygen-transporting capillary beds surrounding B cell follicles in the outer cortex and in the T cell zone. The capillary beds lead directly (or indirectly *via* arteriovenous shunts) into the postcapillary venular network, which extends throughout the paracortex (T cell area) of the LN; HEVs form part of this postcapillary network (**Figure 1**). HEVs are readily distinguished from other blood vessels; the cuboidal (high) ECs, which line HEV and give these vessels their name, contrast with flat ECs lining other vessels. In addition, HEVs are supported by a thickened basal lamina comprising overlapping pericytes and a perivascular sheath of FRC that together generate a structural and functional unit exquisitely adapted to support high levels of lymphocyte recruitment and transendothelial migration to deliver lymphocytes to the LN parenchyma. The ECs lining HEVs express a number of pan-endothelial markers such as VE-cadherin and CD31 as well as the master venous regulator Nr2f2 (69), which suggests that they differentiate from local postcapillary venules. In vivo imaging of lymphocyte recruitment allows HEVs to be ordered according to size and location. The smallest vessels (order V) are found at the junction between the B cell-enriched cortex and the T cell-enriched paracortex. HEVs gradually increase in size to larger vessels (order II) at the junction between the paracortex and medulla where they merge into order I collecting venules which drain into hilar vein (70) (**Figure 1**). The majority of lymphocytes recruited into LN and PPs are from the higher order vessels III–V. In peripheral LN, L-selectin dependent recruitment from lower order vessels does occur but the vascular ligand on HEC is distinct from PNAd (71). How this



complex branching network of differentiated HEVs develops and is maintained in LN is not clear but it is likely to be connected with development of the highly organized secondary structures of FRC-coated conduits that connect the draining lymphatics with the HEVs (29). The dominant roles of LT $\beta$ R signaling in maintaining fully differentiated PNAd-expressing HEVs as well as T/B compartmentalization in LNs are likely to cooperate in forming an organized HEV network.

The ability to image the behavior of leukocytes inside post-capillary venules using intravital microscopy has identified the molecular interactions between lymphocytes and HEVs in LN and PPs that control recruitment from flowing blood. The multi-step adhesion cascade describes the sequence of tethering, rolling and activation-induced arrest, which selects lymphocytes for transendothelial migration and entry into lymphoid organs. The cascade is best exemplified by the fact that neutrophils undergo L-selectin-dependent rolling in HEVs but they are unable to undergo activation-induced arrest by CCL21 immobilized on the inner HEV surface because they do not express CCR7 and are, therefore, not recruited into LN under homeostatic conditions (70). The selectin, chemokines, and integrins that regulate rolling and activation-induced arrest of naive and central memory T and B cells and the non-random recruitment of T and B lymphocytes in HEV of LN and PP of mice have been described in detail elsewhere (1, 3, 72–74). The regulated expression of peripheral node and mucosal addressins by HEV and their roles in lymphocyte recruitment are conserved in LNs of larger animals as well as humans indicating that studies in mice have clinical relevance (75–79). This review will summarize recent advances in understanding the structure and function of HEV and its role in regulating adaptive immunity.

In LNs, L-selectin mediates tethering and rolling of lymphocytes. In comparison with T cells, B cells express lower L-selectin and are reduced in LNs but highly enriched in PPs. Although L-selectin supports lymphocyte rolling in HEVs of PPs, it is not a dominant homing molecule under homeostatic conditions since, in contrast to peripheral LN, the cellularity of PPs in L-selectin deficient mice is not reduced (80). The B-cell expressed lectin that supports preferential recruitment by HEVs of PPs was unknown until a detailed comparison of transcriptomes expressed by HECs isolated from PPs and peripheral LN of mice demonstrated preferential expression of the enzyme  $\beta$ -galactoside  $\alpha$ -2,6-sialyltransferase I (ST6Gal 1) by PP HECs. ST6Gal 1 generates high affinity  $\alpha$ -2,6-sialylated glycan ligands for the B cell lectin CD22 (Siglec-2) and was shown to function as a B cell selective mucosal addressin in PPs (69).

Apart from addressin expression and their location inside LN, a characteristic histological feature of HEV is lymphocytes embedded in the walls of HEV, which suggests that transmigration is a rate-determining step in lymphocyte recruitment from the bloodstream into the LN parenchyma. Transmigration is a rapid event taking 3 min to cross the endothelial lining and 10 min to complete migration across the underlying basal lamina (81–83). The molecules and signaling pathways that regulate transmigration are not completely understood. Whether lymphocytes move through the junctions between HECs (paracellular route) or penetrate the EC cytoplasm (transcellular route)

has long been debated (84, 85). It is important to understand how lymphocytes transmigrate the walls of HEV since the potential for bi-directional signaling in lymphocytes and HECs may prime transmigrating lymphocytes for interstitial motility and immunosurveillance. Recent studies have demonstrated that lymphocytes are held in the so-called HEV pockets that are extracellular spaces between HECs and the surrounding pericyte-containing basal lamina (86). The accumulation of transmigrating lymphocytes, particularly T cells, contributes to the height of HECs (81, 87). Residence in HEV pockets provides an opportunity for cellular contacts with HECs or with other transmigrating cells, such as DCs, which may facilitate rapid transmigration in comparison with vessels lined with flat EC (81). It is also possible that antigen could be passed from the basolateral adjoining conduits to DCs inside HEV pockets for presentation to incoming lymphocytes. HEVs are contractile vessels responding to locally released vasoactive agents (88). Arterial pressure and vasoconstriction can regulate the height of HECs measured in histological sections of LN (89). Dynamic changes in shear stress at the vessel wall interface generated by HEV contractility may enhance the capture of lymphocytes from flowing blood.

Lymphocytes form very close, intercellular, gap-like junctions of 2–4 nm with HECs during transmigration (90), which may be the *in vivo* equivalent of the docking structures between lymphocytes and ECs reported *in vitro* (91, 92). ECs lining HEVs express a range of junctional proteins that are found in other vascular beds, including VE-cadherin, CD31, JAM-A, JAM-B, JAM-C, and ESAM-1 (93). Engagement of EC junctional proteins by complementary receptors on lymphocytes may regulate migration across the endothelial lining of HEVs, as shown for leukocytes transmigrating inflamed blood vessels (67). Unlike other types of endothelium, HEV ECs lack tight junctions and vascular specific claudin-5. These distinct structural features may facilitate paracellular transmigration of lymphocytes and/or lymphocyte accumulation inside HEV pockets.

Some progress has been made in dissecting the molecular events driving entry and exit of lymphocytes from HEV pockets and subsequent penetration of the underlying basal lamina to enter the LN parenchyma (94). Lymphocytes are retained in HEV pockets of FTY720 treated mice, and this was thought to be due to lack of space in the LN parenchyma (86). However, as well as blocking lymphocyte exit from LN *via* lymphatics, FTY720 inhibits T cell recruitment from the bloodstream across HEVs (95). An alternative explanation is that incoming T cells drive the exit from HEV pockets and, in the absence of incoming T cells in FTY720 treated mice, lymphocytes do not complete transmigration, as has been shown *in vitro* (96). There are other mechanisms controlling lymphocyte retention in HEV pockets. T cells accumulate in the endothelial lining of HEVs in mice treated with dual specificity MMP/ADAM inhibitors (87), suggesting that exit from HEV pockets may be metalloproteinase-dependent. L-selectin is proteolytically cleaved from T cells as they transmigrate HEVs (97) and lymphocytes expressing a metalloproteinase-resistant mutant of L-selectin take longer to transmigrate the endothelial lining of HEVs (98). Proteolytic shedding of L-selectin following cross-linking by basolaterally expressed ligands such as PNAd may polarize transmigrating T cells, as recently demonstrated in



monocytes (99) and neutrophils (100) and allow directed migration into the LN parenchyma in response to HEC-derived lipids such as autoaxin and lymphoid stromal cell-derived chemokines such as CCL19 and CCL21 (101, 102).

## The Impact of Immunization and Infection on High Endothelial Venules

There are marked changes to HEVs in LNs draining sites of immunization or infection. These include increased blood flow, expansion of the HEV network, and marked changes in expression of homing-associated molecules. Changes in HEV function cooperate to alter the size and composition of the leukocyte infiltrate. In particular, altered expression of homing molecules on HEV enables the recruitment of activated lymphocytes and innate immune cells that are normally excluded by HEVs because they lack expression of L-selectin or CCR7. The impact of altered immune cell recruitment *via* HEV on ongoing immunity is only just starting to be analyzed (94).

Innate immune cells remodel feeding arterioles to increase blood supply and, thereby, increase the delivery of naïve lymphocytes into draining LNs (103). This allows a major fraction of the full repertoire of lymphocytes to pass through antigen-activated LNs within a few days (104). The HEV network grows to accommodate the increase in blood supply and the accompanying increase in lymphocyte trafficking. For example, the total length of HEVs increases threefold from approximately 10–30 cm in antigen-reactive LN in parallel with a threefold size increase of LN volume (105). LT and VEGF family members regulate expansion of the HEV network. LT $\beta$ R activation of FRCs by DCs induces release of VEGF-A, an angiogenic factor that stimulates HEV growth (65, 106). VEGF-A is also produced by activated DCs, which, following injection into the skin, stimulate the growth of HEV in LN draining the site of injection (66).

Other changes to HEV in antigen-challenged LN relate directly to links with afferent lymphatics. For example, the plasticity of HEV revealed by afferent lymphatic ligation (19) or isolation from the LN environment (20) is directly relevant to adaptive immune responses. Antigen administration is accompanied by a temporary shutdown of afferent lymphatics between days 2 and 5 and concomitant dedifferentiation of HEV (107). Interestingly, the loss of luminal PNAd by HEV following antigen administration is accompanied by transient induction of MAdCAM-1 as found following deafferentiation or CD11c<sup>+</sup> cell depletion. By limiting the influx of lymphocytes, the loss of PNAd expression may serve to prevent the dilution of lymphocytes already in contact with antigen-laden DCs and promote the generation of memory cells. The reversion of PNAd-expressing HEV to MAdCAM-1-expressing HEV may allow entry of LTis or other  $\alpha 4\beta 7$  integrin-expressing innate lymphoid cells (108), for repair or remodeling of LN during the later stages of virus infection to restore preinfection architecture (109).

Vascular changes in antigen-stimulated LNs are accompanied by increased growth of afferent lymphatics and remodeling of the FRC network that alters the availability of chemokines for presentation by HEVs. For example, the dramatic reduction in CCL21 and CXCL13 expression by LN stroma following viral or bacterial infection (110) impacts on T and B lymphocyte recruitment

since stromal cell-derived chemokines are presented by HEVs to blood-borne lymphocytes (32). The increased delivery of DCs and other immune cells mobilized from inflamed tissues *via* expanded afferent lymphatics may have, as yet unexplored, effects on HEV function. However, as the immune response subsides, CD11c<sup>+</sup> cells are required to remodel the perivascular FRC sheath surrounding HEVs and restore vascular permeability and HEV function (111). These observations suggest a regulatory loop based on HEV that controls the size of LN, preventing uncontrolled growth of inflamed lymphoid tissue. How the structure of LNs and other secondary lymphoid tissues is restored to the preactivated state is largely unknown. This is likely to be relevant to understanding TLOs that develop in chronically inflamed tissues.

## BLOOD VESSELS, HIGH ENDOTHELIAL VENULES, AND THE DEVELOPMENT OF TLOs

Tertiary lymphoid organs or ELS develop inside non-lymphoid organs. TLOs form in response to chronic immune-mediated inflammation stimulated by persistent antigens such as infection, allograft rejection or ulcerative colitis but also in several autoimmune conditions such as rheumatoid arthritis and Hashimoto's thyroiditis (112). TLOs have been reported by histology in biopsied or surgically removed clinical tissues from the majority of organs in the body including the CNS and atherosclerotic aorta. TLOs are also found associated with cancerous tissues. TLOs are highly organized lymph node-like structures containing discrete T and B-cell rich areas supported by stromal cells that share markers with FRCs and FDCs in LNs. TLOs contain PNAd-expressing blood vessels that resemble structurally distinct HEV in LNs; they are the presumed sites of entry of blood-borne lymphocyte and, therefore, critical to the function of TLOs. PNAd-expressing blood vessels lined by flat ECs are found inside cancer-induced ectopic lymphoid aggregates that are not organized into distinct T/B cell areas. These could represent immature HEV-containing structures in the process of forming TLOs, or TLOs that are disintegrating during the resolution of chronic inflammation (113). Interestingly, PNAd-expressing blood vessels that form following depletion of Foxp3<sup>+</sup> regulatory T cells from cancer-bearing mice are not associated with histologically distinct, lymphoid cell aggregates (114) which indicates that HEV neogenesis can precede lymphoid neo-organogenesis. The development of HEV in the absence of full-blown TLOs correlates with cancer regression in this experimental model highlighting the important role of HEV in controlling immunity to cancers. Interestingly gp38<sup>+</sup> (podoplanin) stromal cells are induced locally by CD11b<sup>+</sup> myeloid cells recruited to inflamed skin of mice, recapitulating the earliest stages of lymphoid stromal cell development, but whether CD11b<sup>+</sup> myeloid cells drive the development HEV and/or TLOs in chronic inflammation is not known (17).

How HEVs form during ongoing chronic diseases is difficult to dissect but insights to the stimuli and signaling pathways that control HEV neogenesis and function have come from experimental studies in mice. In experimental animals, organized lymphocytic infiltrates containing PNAd-expressing blood

vessels develop in exocrine tissue of the pancreas and thyroid in response to ectopic expression of homeostatic chemokines and cytokines that control LN development (115–118) and virus-induced autoantibody production in the salivary gland (119). However, as found in chronic diseases, the size, location, and composition of lymphoid infiltrates that develop at ectopic sites vary depending on the stimulus. For example, when LT $\alpha$  is expressed in pancreatic  $\beta$  cells PNAd expressing blood vessels are found inside the small lymphoid infiltrates that form around some islets (120). Development is independent of endogenous LT $\beta$  and dependent on signaling *via* the type I TNFR. However, the infiltrates comprise mainly memory T cells that express low levels of L-selectin, which may be due to the predominantly abluminal expression of PNAd, which is unable to recruit L-selectin expressing lymphocytes (118). Co-expression of both LT $\alpha$  and LT $\beta$  and consequent LT $\beta$ R signaling in the exocrine pancreas is required to develop large, organized lymphoid aggregates that contain HEV expressing PNAd at the luminal surface and, as in LNs, these ectopic TLOs are highly enriched in L-selectin-expressing T and B lymphocytes. LT $\alpha\beta$  expressing cells other than LTis drive TLO formation, such as T and B cells which upregulate LT $\alpha\beta$  in response to ectopic expression of CCL21 and CXCL13, respectively (116). PNAd expressing, structurally distinct HEV develop within 5 days of transferring T cells to RAG-deficient mice expressing CCL21 under the thyroglobulin promoter and, as found in LN, HEV development is dependent on LT $\alpha\beta$ –LT $\beta$ R signaling and DCs (117, 121). As in LN, the development of PNAd expressing HEV in cytokine or chemokine-induced TLOs is stunted in mice deficient in either LT $\alpha$  or LT $\beta$ . PNAd-expressing blood vessels lack the HEV-restricted sulfotransferase and, therefore, luminal expression of PNAd, and are lined with flat ECs typical of immature HEVs in LN unable to support high levels of lymphocyte traffic. The lack of HEV maturation in the absence of LT $\alpha$  or LT $\beta$  correlates with and, most likely, contributes to the reduced size and cellularity of lymphoid infiltrates in these mice (118).

The development of PNAd-expressing HEV in mouse models of cytokine- or inflammation-induced cancer correlates with increased T cell infiltration and priming and reduced tumor growth (114, 122, 123). In tumor cell transplant models, PNAd expression is induced on tumor blood vessels by infiltrating tumor-specific effector CD8 $^{+}$  T cells as well as NK cells (124). In marked contrast to HEV development in LN and TLO, PNAd expression is not dependent on LT $\beta$ R signaling but is stimulated by CD8 $^{+}$  T and NK cell-derived LT $\alpha$ 3 activation of TNFR. Interestingly, expression of the arrest chemokine CCL21 is not induced by the same stimuli that induce PNAd expression, but instead by IFN- $\gamma$  released by activated T and NK cells. However, the tumor-associated HEVs are distinct from conventional mature HEVs in LN since PNAd expression is exceptionally low and the endothelial lining is flat, rather than the characteristic, LT $\beta$ R-dependent cuboidal morphology found in LNs (50). Although comprising <10% of the tumor vascular network and lined by flat EC, these PNAd-expressing tumor blood vessels are functional in that they recruit naïve, L-selectin-expressing T cells from the bloodstream into the tumor where they are activated to kill tumor tissue (124). These findings suggest that TNFR

signaling in ECs stimulates the development of PNAd-expressing blood vessels resembling immature HEVs in LNs and that these vessels promote anti-tumour immunity by recruiting naïve T cells into cancerous tissues. This allows T cell priming and reactivation inside solid cancers, thus avoiding the dilution of tumoricidal T cells during redistribution from their LN site of priming *via* the efferent lymphatics and bloodstream (123).

The presence of HEV-containing TLO is highly correlated with active disease and for persistent infections such as *H. pylori*, TLOs in the gastric mucosa disappear when the infection is cleared (125). In the case of solid, vascularized cancers, the presence of TLO in resected solid cancers has been correlated with prolonged patient outcome following resection of the primary cancer in breast cancer (126, 127), melanoma (128, 129), lung (130), and colorectal cancer, although in the latter case the correlation depends on the stage of the disease (131, 132). In separate studies of lung cancer, mature DC containing TLO enriched in CD8 $^{+}$  effector memory T cells or expressing a LN-associated chemokine and adhesion molecule gene signature have both been correlated with improved patient outcome (133, 134). Importantly however, the formation of TLO does not always indicate improved cancer patient outcome. In virus-induced hepatic cellular carcinoma, TLOs provide a cytokine-rich niche, which promotes the development and survival of malignant hepatocyte progenitors (11). The density of HEV alone was sufficient to predict patient outcome in breast cancer and melanoma (9, 10), indicating the critical role that HEVs play in orchestrating anticancer immunity. It will be interesting to determine the expression of other markers of HEV differentiation such as MADCAM-1, GlyCAM-1, GlcNAc6ST-2, and GlcNAc6ST-1 to further understand the precise roles of HEV development and maturation in protective immunity to clinical cancers.

Evidence of ongoing immune responses are seen inside TLOs; activation-induced cytidine deaminase is active in ectopic germinal centers in salivary glands of Sjogren's syndrome patients (5) and T cell priming and epitope spreading occur in mouse models of multiple sclerosis and cancer (122, 135). In mice lacking all SLOs, Moyron-Quiroz and colleagues demonstrated that humoral and cellular immune responses develop in TLOs following influenza infection (136). The formation of TLO in clinical conditions may, therefore, reflect a lack of function in draining secondary LNs, which are no longer able to accept incoming antigen or antigen-presenting cells or that SLOs are operating at maximal capacity. The formation of TLOs is thought to exacerbate autoimmune diseases, at least in part, because effector lymphocytes generated within the target organ will not be diluted out during transit from the normal LN site of priming. However, the impact of TLO will depend on the nature of the ongoing immune response to the autoantigen, pathogen, or cancer antigen, which may be protective (CD8 $^{+}$  T cells in cancer), inflammatory (Th17 cells in autoimmunity), or tolerance inducing (Foxp3 $^{+}$  regulatory T cells), and this may be regulated by the activation status of PNAd-expressing HEVs and additional factors such as chemokines. Further studies are required to understand exactly how ectopic HEVs are formed and their impact on different types of chronic diseases. Such studies may reveal therapeutic targets for intervention in autoimmune diseases and cancers.

## A Unifying Hypothesis of HEV Neogenesis

Extensive studies of SLOs and TLOs have shown that HEVs present as peripheral and/or mucosal addressin expressing blood vessels in which the endothelium is either characteristically cuboidal and filled with transmigrating lymphocytes or flat and, although PNAd-positive, lymphocyte-filled pockets are absent. Can we reconcile these divergent reports of HEV into a single, unifying model of HEV neogenesis in lymphoid and non-lymphoid tissues? Clues for this have come from studies in which cytokines ectopically expressed in pancreatic islets of mice stimulate the development of vascular addressin expressing blood vessels. Expression of LT $\alpha$ -induced PNAd and MAdCAM-1 on pancreatic blood vessels dependent on signaling through TNFR1 but the ECs are flat, do not express the HEV-restricted sulfotransferase (HEC-6ST/GlcNAc6ST-2) and PNAd expression was located to the basolateral EC surface where, in LN, it does not support high levels of lymphocyte recruitment. Structurally distinct, PNAd-expressing blood vessels, similar to HEVs in LN, were only formed when LT $\alpha$  and LT $\beta$  were co-expressed in pancreatic islets (137). Interestingly, LT- $\alpha$  drives the development of flat, PNAd-expressing blood vessels in mouse models of cancer which, although structurally similar to immature HEVs in LN, are able to recruit L-selectin-expressing T cells from the bloodstream *via* PNAd<sup>+</sup> ligands indicating they are not completely immature (124).

LT $\alpha$  and LT $\alpha\beta$  activate the classical NF- $\kappa$ B pathway characterized by nuclear translocation of p50-RelA complexes. LT $\alpha\beta$  also activates the alternative, non-canonical NF- $\kappa$ B pathway of NF- $\kappa$ B-inducing kinase (NIK)-dependent activation of I $\kappa$ B kinase (IKK)- $\alpha$  and nuclear translocation of p52-RelB complexes (138). Non-canonical NF- $\kappa$ B signaling plays a dominant role in the formation of HEVs in LNs since blockade of LT $\beta$ R, but not TNFR, leads to loss of several HEV-specific markers such as GlyCAM-1, MAdCAM-1, CCL21, and the HEV-restricted sulfotransferase, HEC-6ST/GlcNAc6ST-2 (58). In addition, PNAd-expressing blood vessels that develop in IKK $\alpha$ (AA) mutant mice where non-canonical NF- $\kappa$ B signaling is defective lack GlyCAM-1 and HEC-6ST (49). Conversely, mice lacking full-length p100 protein, resulting in constitutively active p52, develop PNAd-positive HEVs in the spleen (139). The key event in non-canonical NF- $\kappa$ B activation is signal-induced protein stabilization of NIK that is normally degraded by a ubiquitin ligase complex comprising TRAF2, TRAF3, and cIAP1/2 (140, 141). LT $\beta$ R ligation sequesters this NIK-targeting destruction complex leading to NIK accumulation. Importantly, signal-induced NIK stability is transient (142), suggesting that continual activation of the LT $\beta$ R is required to maintain functional NIK expression levels and hence, sustain the activity of the non-canonical NF- $\kappa$ B pathway.

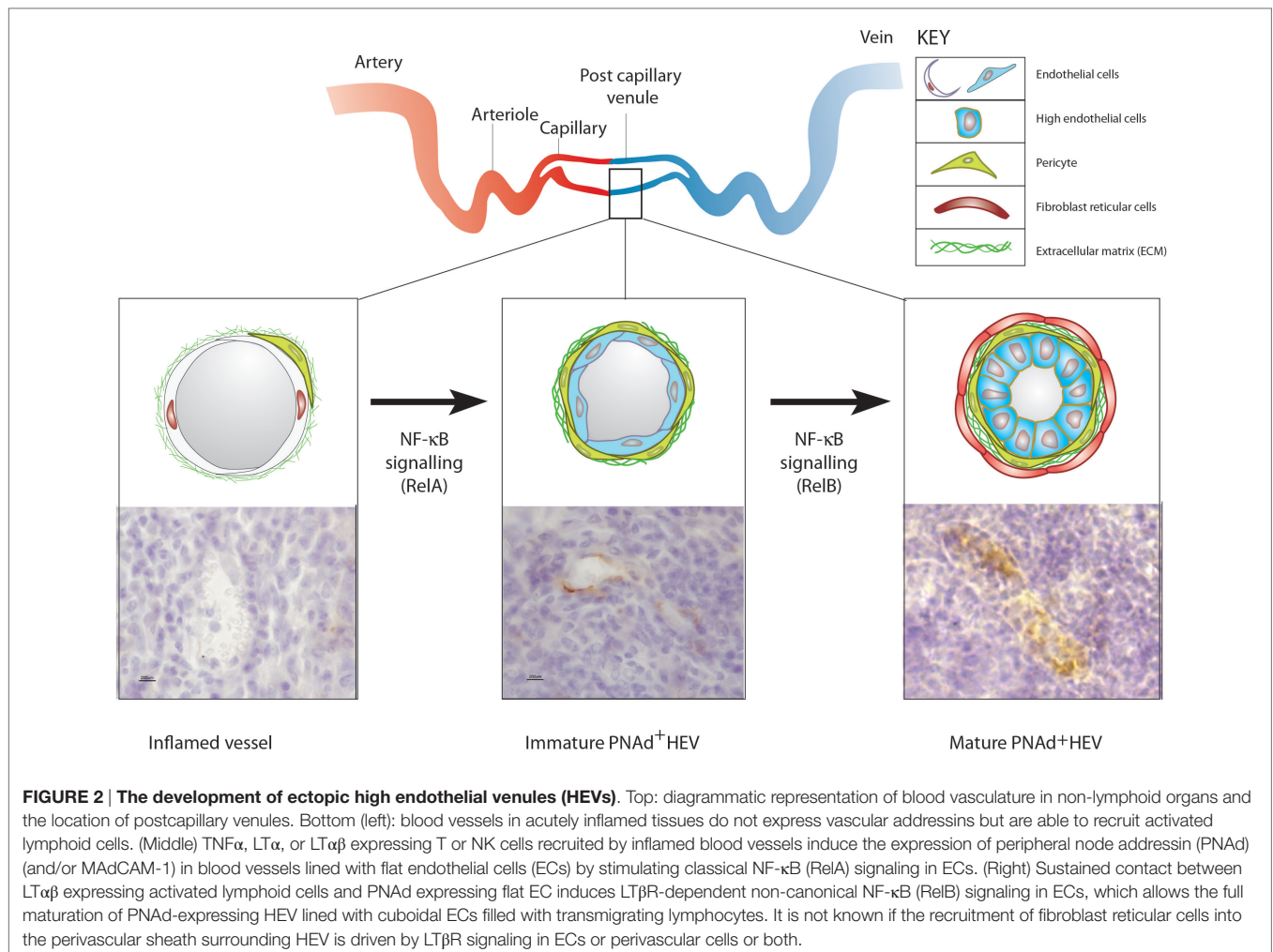
It is proposed that the development of HEVs is dissected into at least two distinct stages based on NF- $\kappa$ B signaling in blood vessel ECs (**Figure 2**). The first stage is driven by LT $\alpha$ -TNFR classical NF- $\kappa$ B signaling and generates MAdCAM-1-expressing HEV lined with flat, PNAd-expressing ECs. The second stage is driven by sustained LT $\beta$ R non-canonical NF- $\kappa$ B signaling and induces the development of fully mature, PNAd-expressing HEVs lined by HECs and containing lymphocyte-filled pockets.

However, these two stages may be not always be clearly delineated because there is overlap between classical and non-canonical NF $\kappa$ B signaling in blood ECs. For example, classical RelA/NF- $\kappa$ B signaling by TNF $\alpha$ , LT $\alpha$ , or LT $\alpha\beta$  in EC cultured from human and mouse tissues induces expression of MAdCAM-1 protein and expression of the gene encoding HEC-6ST, which generates the PNAd epitope in LN HEVs (33–35, 143). However, LT $\beta$ R signaling in isolated human EC has not been reported to induce the expression of PNAd modified glycoproteins (143). It is known that classical NF- $\kappa$ B-dependent signaling in human EC inhibits non-canonical NF- $\kappa$ B signaling (144). The outcome of LT $\beta$ R signaling in ECs will, therefore, depend on the balance between classical and non-canonical NF- $\kappa$ B signaling. Recent studies have demonstrated that endothelial differentiation is regulated by components of the basal lamina (145). As in other types of postcapillary venule, structural support to the endothelial lining of HEV is provided by the basal lamina, which is known to regulate NF- $\kappa$ B signaling (146). Further studies on isolated blood ECs may identify the stimuli and signaling pathways that stimulate the synthesis of HEV-restricted PNAd-modified glycoproteins thereby controlling HEV neogenesis.

## THERAPEUTIC STRATEGIES TO CONTROL HEV NEOGENESIS AND FUNCTION

If HEVs in TLO are critical to exacerbation of autoimmune diseases by allowing activation of tissue destroying lymphocytes within target tissues, can the development of HEVs be prevented? Since HEVs in SLOs are important for generating protective immunity to infection any such therapy would need to be targeted to TLO. Intuitively, this will require the identification of markers or signaling pathways in ectopic HEVs that are not shared by HEVs in LN. One candidate is TNFR signaling that is not required for the development or maintenance of HEVs in LN (58) but has been shown to induce PNAd-expressing ectopic HEVs able to recruit naïve T lymphocytes, at least in cancer (124). It is possible that the success of anti-TNF- $\alpha$  or TNFR1I-Ig (Etanercept) therapies in rheumatoid arthritis patients may depend, in part, on reversing or blocking blood vessel differentiation toward an HEV-like phenotype. The formation of HEVs in cancerous tissues in the absence of TLO correlates with reduced tumor progression in experimental animals; HEV neogenesis may, therefore, be a possible therapy to control cancer growth but it is not clear how this would be achieved. Clinical and experimental data indicate that tumor blood vessels are poor at recruiting cytotoxic, effector T lymphocytes and present an immune checkpoint that limits effective immunotherapy (147). Several different strategies are being considered to achieve this including targeted delivery of TNF- $\alpha$  to tumor blood vessels, which may induce the development of PNAd-expressing blood vessels although this was not determined (148). Approaches to induce HEV-containing TLO formation in cancers are also being considered but it is worth bearing in mind that TLOs, like SLOs, could be sites of tolerance induction and, therefore, may limit effective antitumor immunity. The full impact of HEVs and/or TLO may only be revealed when





highly immunosuppressive cells such as  $\text{Foxp3}^+$  Tregs or myeloid-derived suppressor cells are depleted allowing effector T cells to exit TLO, infiltrate, and kill cancerous tissues (114, 149, 150). It will be important to determine which immune cells are recruited by cancer-associated HEVs to dissect their impact on cancer immunity. Further studies are required to determine the extent and role of HEV development in clinical cancers.

## AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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